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Brahma regulates the Hippo pathway activity through forming complex with Yki–Sd and regulating the transcription of Crumbs

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ABSTRACT

The Hippo signaling pathway restricts organ size by inactivating the Yorkie (Yki)/Yes-associated protein (YAP) family proteins. The oncogenic Yki/YAP transcriptional coactivator family promotes tissue growth by activating target gene transcription, but the regulation of Yki/YAP activation remains elusive. In mammalian cells, we identified Brg1, a major subunit of chromatin-remodeling SWI/SNF family proteins, which interacts with YAP. This finding led us to investigate the *in vivo* functional interaction of Yki and Brahma (Brm), the *Drosophila* homolog of Brg1. We found that Brm functions at the downstream of Hippo pathway and interacts with Yki and Scalloped (Sd) to promotes Yki-dependent transcription and tissue growth. Furthermore, we demonstrated that Brm is required for the Crumbs (Crb) dysregulation-induced Yki activation. Interestingly, we also found that *crb* is a downstream target of Yki–Brm complex. Brm physically binds to the promoter of *crb* and regulates its transcription through Yki. Together, we showed that Brm functions as a critical regulator of Hippo signaling during tissue growth and plays an important role in the feedback loop between Crb and Yki.

1. Introduction

The regulation of cell-fate determination and growth is central to the animal tissue homeostasis. The Hippo signaling pathway is an emerging growth control pathway that is conserved from *Drosophila* to mammals [1,2]. In *Drosophila*, the core kinase complex of Hippo pathway includes Ste20-like kinase Hippo (Hpo) and NDR family kinase Warts (Wts), as well as the adaptor proteins, including Salvador (Sav) and Mob as tumor suppressor (Mats) [3]. When Hpo is activated by upstream regulators, the active Hpo subsequently phosphorylates and activates Wts, which phosphorylates the transcriptional coactivator Yorkie (Yki), restricting its distribution in the cytoplasm by promoting its interaction with 14-3-3. When Hpo is inactivated, unphosphorylated Yki translocates into the nucleus and interacts with the TEAD/TEF family transcription factor Sd (Scalloped) to trigger the expression of target genes including myc, cyclin E, Diap1, and bantam, leading to cell proliferation and anti-apoptosis [3,4]. In mammals, the inhibition of Hippo pathway results in the activation of Yes-associated protein (YAP) or transcriptional coactivator with PDZ binding motif (TAZ), two orthologs of Yki. YAP/TAZ binds to the TEAD family transcription factors to promote organ growth [4]. Defects in Hippo signaling and hyperactivation of YAP/TAZ contribute to the occurance of numerous types of human cancer [5,6].

Although the core signaling cascade of Hippo pathway has been extensively studied, the regulation of the Hippo pathway remains poorly understood. To date, there are four interconnected upstream branches regulating the Hippo pathway: apical–basal polarity proteins, cell–cell junctions, actin cytoskeleton and other regulators intersect with the core kinase cascade (reviewed in [1,3,4]). Recently, the interactors of Hpo signaling, including GAGA factor, MASK and Tgi/Vgll4, have been identified as the modulators of Yki/YAP-mediated transcription [7–12], which suggest that there could be more unknown regulatory mechanisms underlying the Hpo signaling. In order to identify the potential regulators of Yki/YAP activation, we performed tandem affinity purification (TAP) and mass spectrometry analysis in HeLa cells using TAP-tagged YAP as a bait. Among the interacting proteins, we identified Brahma-related gene 1 (Brg1) as a YAP interacting protein.

Brg1 is a core subunit of the SWItch/sucrose nonfermentable (SWI/SNF) complex, which is an evolutionarily conserved and well characterized as an ATP-dependent chromatin-remodeling complex. SWI/SNF complex functions both in transcription activation and gene silencing by modulating nucleosome positioning [13]. SWI/SNF complex-mediated transcriptional regulation have been highly implicated in controlling

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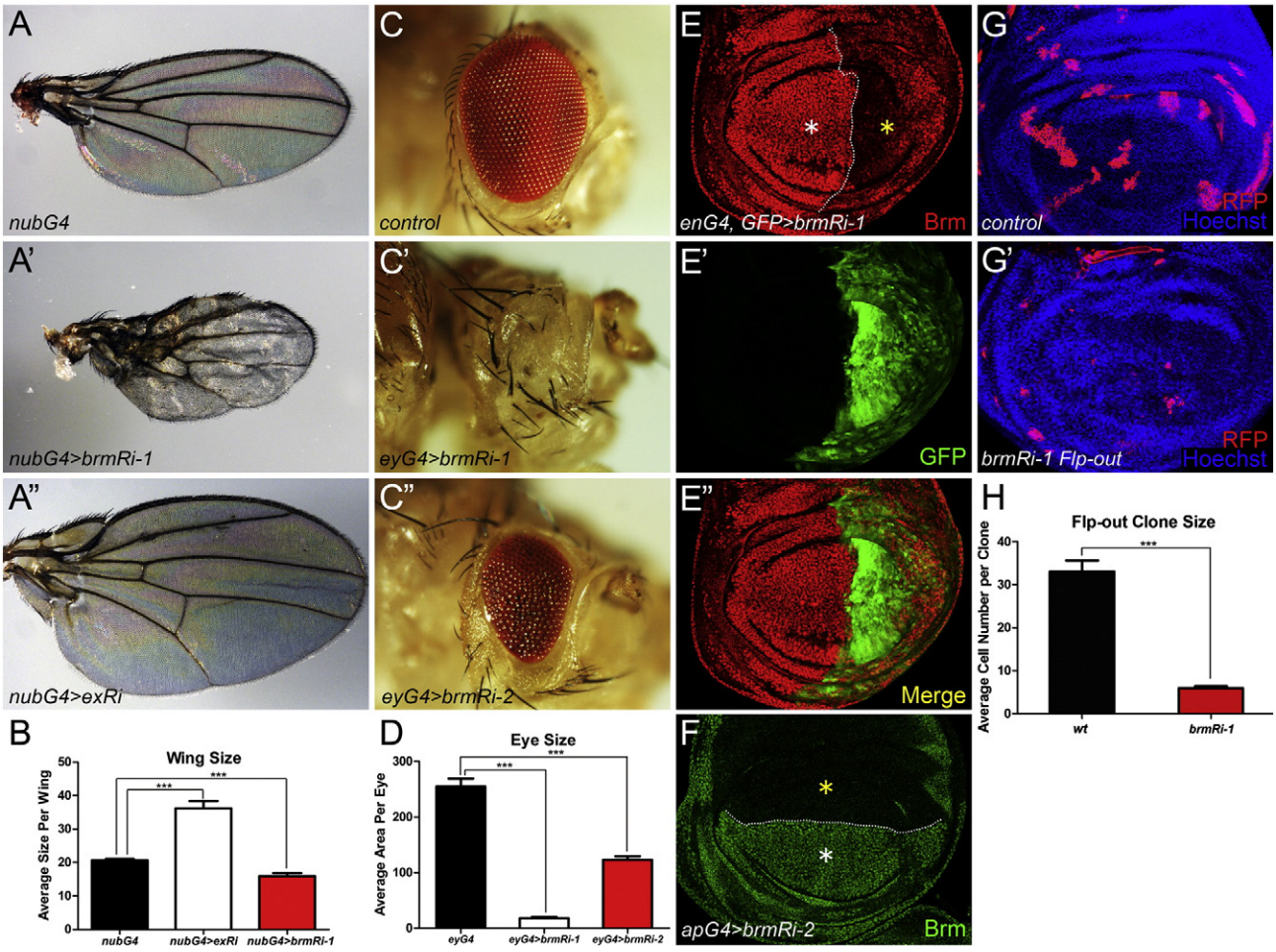

Fig. 1. The *Brm* gene is required for tissue growth in the *Drosophila* wing and eye. (A–C″) The genetic crosses were performed at 18 °C, unless otherwise indicated, all of the flies were cultured at 25 °C. (A) Adult wing of *nubGal4*. (A′) Knockdown of *Brm* resulted in an abnormally small wing. (A″) Knockdown of *ex* resulted in an abnormally large wing. (B) Quantification of adult wing sizes ($n = 4$ for each genotype, $^{***}P < 0.001$). (C) Adult of *eyGal4*. (C′–C″) *UAS-brmRNAi* transgenes were driven by *eyGal4*. Both resulted in abnormal small eyes. (D) Quantification of adult eye sizes ($n = 3$ for each genotype, $^{***}P < 0.001$). (E–E″) *enGal4, UAS-GFP > brmRi-1* wing disc stained for Brm. Yellow asterisk note the lower levels of Brm. (F) The knockdown efficiency of *brmRi-2* was detected by Brm antibody staining (indicated by yellow asterisk). (G–G′) Third instar wing disc containing flp-out clones (G′, RFP-positive) expressing *brmRi-1* that are drastically smaller than that in control (G). Nuclei (blue) stained by Hoechst 33258. (H) Quantification of the clone sizes between the control (G) and *brmRi-1* (G′). Upon *brm* knockdown, cell number per clone (6 ± 0.415 versus 33 ± 2.444, $n = 50$, $^{***}P < 0.001$,) decreased for 5.5 folds.

mammalian stem cell self-renewal and differentiation [14–19]. Consistently, recent findings have revealed the role of Brm complex in regulating stem cell pluripotency in *Drosophila* brain and mid-gut [20–22].

In the present study, we utilized the *Drosophila* model to characterize the role of Brm in the regulation of Hpo signaling during tissue growth, and we found that Brm functions as the downstream of Hippo pathway and interacts with Yki and Scalloped (Sd), which promotes Yki-dependent transcription and tissue growth. Since it has been shown that the apical determinant Crumbs (Crb) functions as an important upstream input for the regulation of Hippo signaling [23–26], we defined that Brm is required for the Crb dysregulation-induced Yki activation. Finally, we found that *crb* is a downstream target of Yki–Brm complex. Together, we showed that Brm functions as a critical regulator of Hippo signaling during tissue growth and plays an important role in the feedback loop between Crb and Yki.

2. Materials and methods

2.1. Drosophila strains

The following fly lines were used in this study: *nubGal4, eyGal4, enGal4, hhGal4, apGal4* and *GMRGal4* were as described in FlyBase. ex^{AP49}, *ex-lacZ*, crb^{11A22} and *UAS-*crb^{intra} were gifts from G. Halder [23,27]. hpo^{BF33} [28], *bantam sensor* [29], *UAS-yki*, *diap1-GFP* [30] and *UAS-brm* [21] were gifts from L. Zhang. *dpp-lacZ* [31], *wg-lacZ* [32], and *ywhsflp; act>*y^{+}*>Gal4, UAS-CD8-RFP/Cyo* were gifts from X. Lin. *UAS-brmRNAi-1* (HMS00050), *UAS-brmRNAi-2* (HM04019) and *UAS-exRNAi* (HMS00874) were obtained from the *Drosophila* RNAi Screen Center at Harvard Medical School.

2.2. Generation of mutant clones

Fig. 1G: *ywhsflp/+; act>*y^{+}*>Gal4, UAS-CD8-RFP/+; +/+*
Fig. 1G′: *ywhsflp/+; act>*y^{+}*>Gal4, UAS-CD8-RFP/+; UAS-brmRNAi-1/+*
Fig. 2A: *+/+;* ex^{AP49}, y^{+}, FRT^{40A}*/uGFP* FRT^{40A}*; eyGal4, UAS-flp/+*
Fig. 2A′: *+/+;* ex^{AP49}, y^{+}, FRT^{40A}*/uGFP* FRT^{40A}*; eyGal4, UAS-flp/UAS-brmRNAi-2*
Fig. 2B: *+/+;* FRT^{42D} hpo^{BF33}*/*FRT^{42D} *uGFP; eyGal4, UAS-flp/+*
Fig. 2B′: *+/+;* FRT^{42D} hpo^{BF33}*/*FRT^{42D} *uGFP; eyGal4, UAS-flp/UAS-brmRNAi-2*
Supplementary Fig. 3A–A″: *+/+; enGal4, UAS-flp/+;* FRT^{82B} *e,* crb^{11A22}*/*FRT^{82B} *uGFP*

2.3. Immunostaining and microscopy

The following primary antibodies were used: rabbit anti-Brm and rabbit anti-Yki (gift from L. Zhang) [21], mouse anti-Crb (Cq4, DHSB), mouse anti-Wg (4D4, DHSB), guinea pig anti-Sens and rabbit anti-

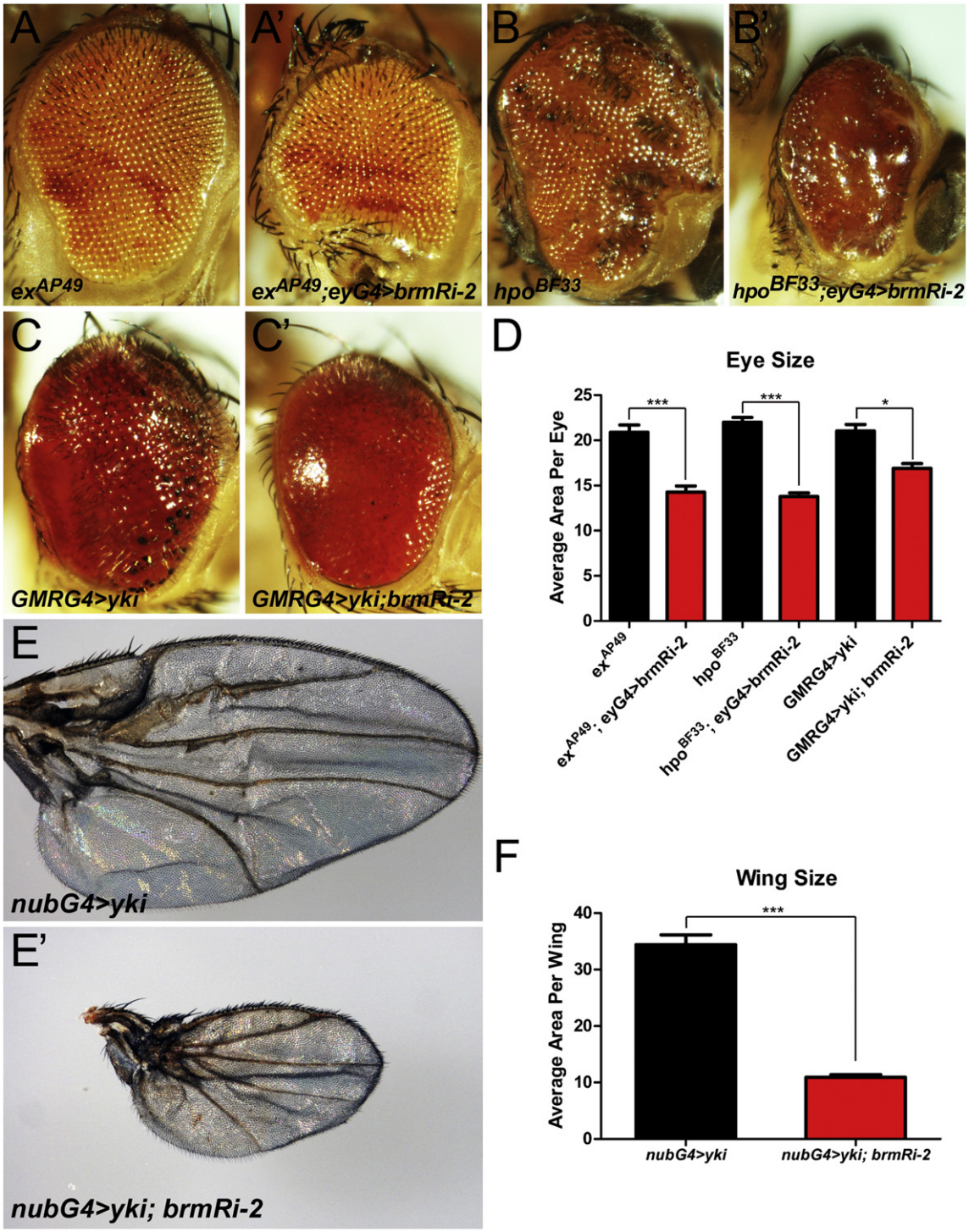

Fig. 2. Brm genetically interacts with Hippo pathway components. The crosses, except C and C′, were performed at 18 °C. C and C′ were performed at 25 °C. (A) Side view of *ex* mutant eye. (A′) Depletion of *brm* in the *ex* mutant background. (B) *hpo* mutant eye. (B′) Depletion of *brm* in the *hpo* mutant background. (C) Yki gain-of-function eye driven by *GMRGal4*. (C′) *UAS-yki* and *UAS-brmRNAi-2* were co-expressed using *GMRGal4*. (D) Quantification of adult eye sizes ($n = 5$ for each genotype, $^{***}P < 0.001$, $^{*}P < 0.05$). (E) Overexpression of *yki* resulted in an abnormal large wing. (E′) Depleting *brm* repressed the enlarged-wing caused by *yki* overexpression. (F) Quantification of adult wing sizes ($n = 3$ for each genotype, $^{***}P < 0.001$).

Sal (gift from X. Lin), mouse anti-Ptc (DSHB), rat anti-Ci (2A1; DSHB), mouse anti-LacZ (Abmart) and rabbit anti-GFP Alexa Fluor 488 (Molecular Probe). The nucleuses were stained by Hoechst 33258 (Sigma). The fluorescent-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. Confocal images were collected using a Lecia TCS SP5 confocal microscope with 40X/1.25 oil objectives. Adult wing and eye images were obtained using a Nikon SMZ1500 microscope.

2.4. Cell culture, transfection and western blot

HEK293T cell line was cultured in DMEM supplemented with 10% fetal bovine serum (Gibco), 50U/ml penicillin, 50 μg/ml streptomycin, in 5% CO_2 atmosphere at 37 °C. Plasmid transfection was carried out using LipofectAMINE (Invitrogen). The *pML-Gal4* and *arm-Gal4* plasmids were gifts from X. Lin. The plasmids *UAS-Myc-yki*, *UAS-Flag-brm* and *UAS-HA-sd* were gifts from L. Zhang [21]. The primary antibodies

used for Immunoprecipitation and western blot were rabbit anti-Brm and rabbit anti-Yki [21], mouse anti-Crb (Cq4, DHSB), mouse anti-Tubulin (CWBiotech), mouse anti-Myc (Santa Cruz Biotechnology), mouse anti-Flag (Invitrogen), mouse anti-HA (Santa Cruz Biotechnology) and mouse anti-GAPDH (CWBiotech).

2.5. Chromatin immunoprecipitation (ChIP) assay

S2 cells were fixed for 15 min in 1% formaldehyde/PBS. The cells were lysed (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1 % SDS and protease inhibitors) and sonicated on ice. Chromatin was immunoprecipitated with anti-Flag (Sigma) or anti-Myc (Santa Cruz) antibody. Complexes were pulled down with Protein A Dynabeads (Invitrogen), washed four times with washing buffer (50 mM HEPES pH 8.0, 0.5 M LiCl, 1 mM EDTA, 0.7% sodium deoxycholate, 1% NP-40) and once with TE. The DNA was eluted, decross-linked for 6 h at 65 °C and purified using the QIAquick PCR purification kit (QIAGEN). DNA was analyzed by real-time qPCR, and enrichment was calculated for each antibody relative to input DNA. The primer sequences are as follows:

crb forward, 5′-TAAAATCGCCAATGCGTCAC-3′;
crb reverse, 5′-GCTTTTCGTGCGGTCTCG-3′.
rp49 forward, 5′-CGTTTACTGCGGCGAGAT-3′;
rp49 reverse, 5′-CCGTTGGGGTTGGTGAG-3′.

2.6. Statistical analysis

Statistical analysis of the data was performed with a two-tailed Student's *t*-test or one-way ANOVA followed by Fisher's PLSD (protected least significant difference) post hoc test using the Origin software (Version 8). Data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ denotes statistical significance.

3. Results

3.1. Brm, the Drosophila homolog of Brg1, is required for tissue growth

In our TAP assay in which FLAG-HA-tagged YAP was used as a bait, we found Brg1 had a high binding affinity with YAP (data not shown), indicating that Brg1 is a potential regulator of Hippo signaling. To investigate the functional interaction *in vivo*, we first studied the function of Brm, the *Drosophila* homolog of Brg1, in regulating tissue growth. The knockdown of *brm* using the *nubGal4* driver resulted in a strong

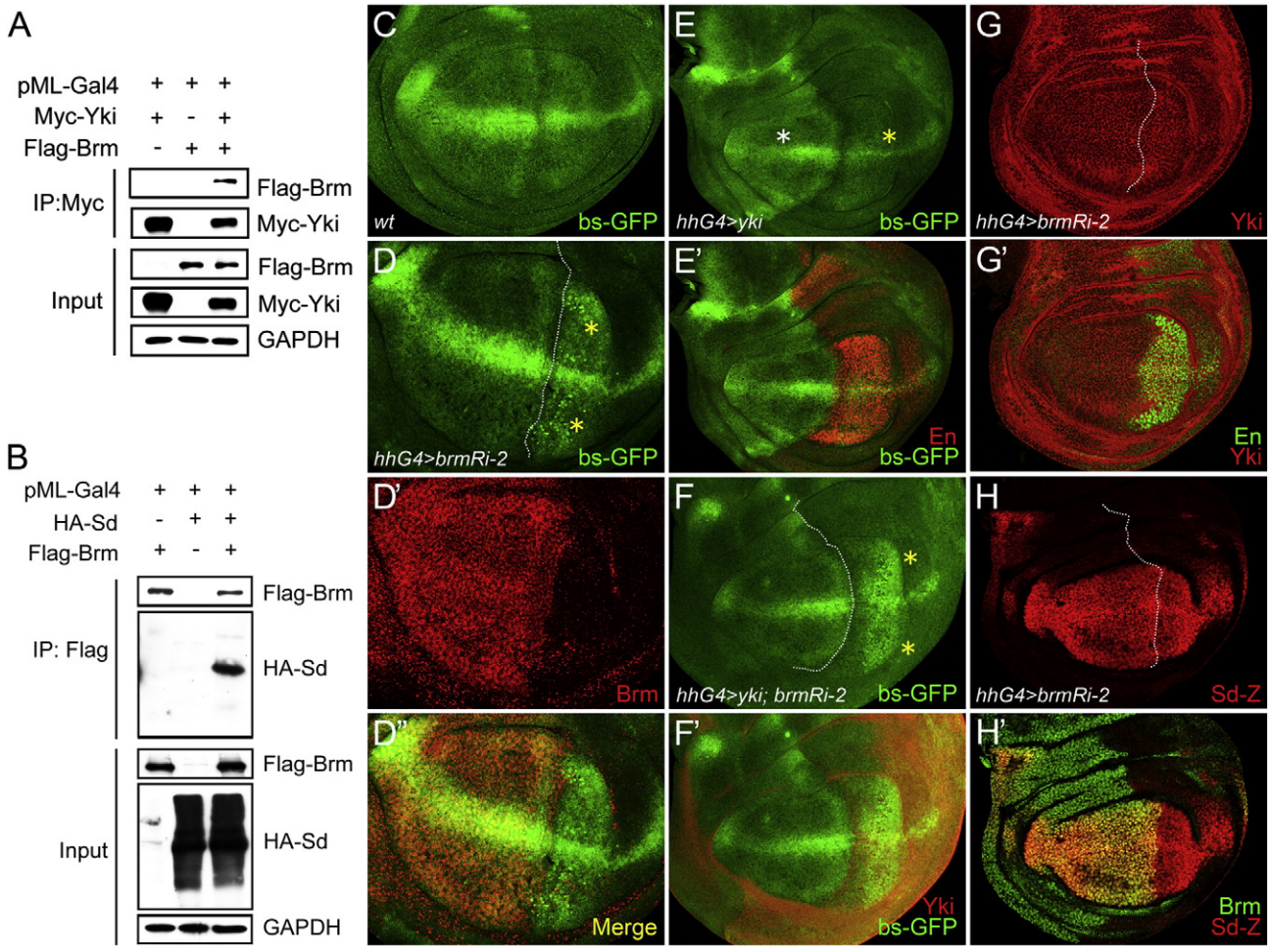

Fig. 3. Brm complexes with Yki and Sd and regulates Yki activity. (A) Lysates of HEK293T cells transfected with *pML-Gal4* together with *UAS-Myc-yki* and *UAS-Flag-brm* were immunoprecipitated with anti-Myc antibody. Western blot analysis was performed using anti-Flag or Myc antibody. 2% input was blotted with anti-Flag, HA or GAPDH antibodies, respectively. (B) Lysates of HEK293T cells transfected with *pML-Gal4* together with *UAS-HA-sd* and *UAS-Flag-brm* were immunoprecipitated with anti-Flag antibody. Western blotting was performed with the antibodies indicated. (C) *bantam-sensor* (*bs-GFP*) control. (D–D″) knockdown of *brm* in the P compartment by *hhGal4* increased the levels of *bs-GFP* (indicated by yellow asterisks). (E–E′) Overexpression of yki by *hhGal4* driver reduced the levels of *bs-GFP* in the P compartment (indicated by yellow asterisk). The P compartment was indicated by Engrailed (En) staining. (F–F′) Coexpression of *brmRi-2* and *yki* reversed the reduction of bs-GFP caused by *yki* overexpression. (G–G′) Knockdown of *brm* did not affect the Yki protein levels indicated by Yki antibody staining (G). (H–H′) The transcription of *sd* indicated by Sd-LacZ reporter, was not influenced by Brm knockdown (H).

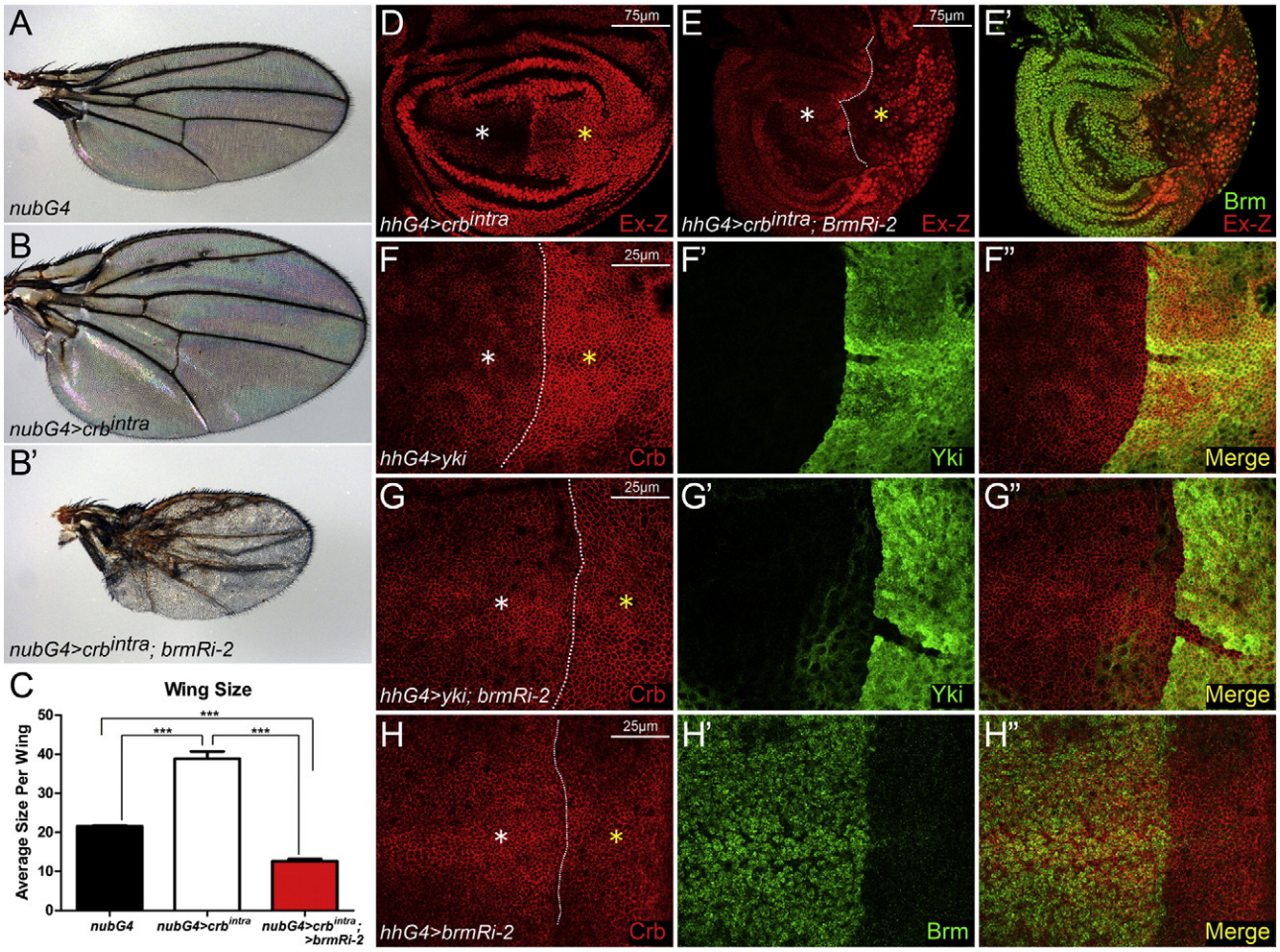

Fig. 4. Crb requires Brm for regulation of Hippo target gene and is a downstream target of Yki–Brm complex. (A–E′) The crosses were performed at 18 °C. (A) adult wing of *nubGal4*. (B–B′) Overexpression of crb^{intra} caused overgrowth, which was suppressed by depleting *brm* (shown in B′). (C) Quantification of adult wing sizes ($n = 4$ for each genotype, $^{***}P < 0.001$). (D–E′) Wing discs that expressed crb^{intra} (D), and crb^{intra} and *brmRi-2* (E) in the P compartment driven by *hhGal4*. crb^{intra} expression caused the upregulation of *ex-lacZ* (yellow asterisk in D), whereas coexpression of *brmRi-2* with crb^{intra} reduced the *ex-lacZ* induction (yellow asterisk in E) and the compartment size. Dotted lines point to the compartment boundaries. (F–F″) Overexpression of *yki* using *hhGal4* elevated the proteins levels of Crb in the P compartment (yellow asterisk in F). (G–G″) The upregulation of *crb* caused by *yki* overexpression was suppressed by *brm* knockdown (yellow asterisk in G). (H–H″) Depletion of *brm* by *hhGal4* reduced the proteins levels of Crb in the P compartment (yellow asterisk in H).

growth-reduced phenotype in the wings, which is opposite to the phenotype of *ex (expanded)* knockdown (Fig. 1A and B). Similarly, the knockdown of *brm* using the *eyGal4* resulted in smaller eyes (Fig. 1C and D). We repeated this experiment using another RNAi (*brmRi-2*) and obtained a similar phenotype (Fig. 1C″). The knockdown efficiency of two RNAi lines was confirmed by immunostaining (Fig. 1E and F). In the developing larval wing disc, *brm*-depleted clones displayed poor proliferation and significant reduced size (Fig. 1G and H). These data indicate that Brm is essential for cell proliferation and tissue growth.

3.2. Genetic interaction of Brm and Hippo pathway

In order to determine the functional link between Brm and Hippo signaling components, we first investigated their genetic interactions. Loss-of-function mutations of *ex* or *hpo* resulted in tissue overgrowth phenotype (Fig. 2A and B). Brm knockdown dramatically inhibited the *ex* or *hpo* mutation-induced overgrowth in adult eyes (Fig. 2A′ and B′, D), suggesting that tissue overgrowth caused by loss-of-function mutations of *ex* and *hpo* required Brm. The overexpression of *yki* by *GMRGal4* driver resulted in rough eye phenotype and Brm knockdown repressed the phenotype (Fig. 2C–C′, D). Consistently, the overexpression of *yki* driven by *nubGal4* led to overgrown adult wings and knockdown of *brm* strikingly suppressed the phenotype (Fig. 2E and F). Together, these results suggest that Brm acts at the downstream of Yki to regulate tissue growth.

3.3. Brm forms complex with Yki and Sd and is required for Yki activity in vivo

Consistent with the previous observation of Jin et al. [21], we also found that Brm forms complex with Yki and Sd (Fig. 3A and B). Next, we examined whether Brm is required for the expression of Yki target genes. The knockdown of *brm* led to a significant enhancement in the expression of *bantam-sensor* reporter (*bs-GFP*) (Fig. 3D–D″). In *yki* overexpression cells, the levels of *bs*-GFP were dramatically reduced (Fig. 3E–E′). This reduction can be reversed by coexpression of *UAS-brmRNAi* (Fig. 3F–F′). Additionally, the overexpression of *yki* increased the expression of *diap1-GFP* (Supplementary Fig. 1A–A″). The depletion of *brm* dramatically suppressed the expression of diap1-GFP caused by *yki* overexpression (Supplementary Fig. 1B). Altogether, these results show that Brm is required for the expression of Yki target genes. Then we asked whether the expression of Yki or Sd was regulated by Brm. We found that neither the protein level of Yki (Fig. 3G–G′) nor the transcription of Sd indicated by Sd-lacZ reporter (Fig. 3H–H′) was

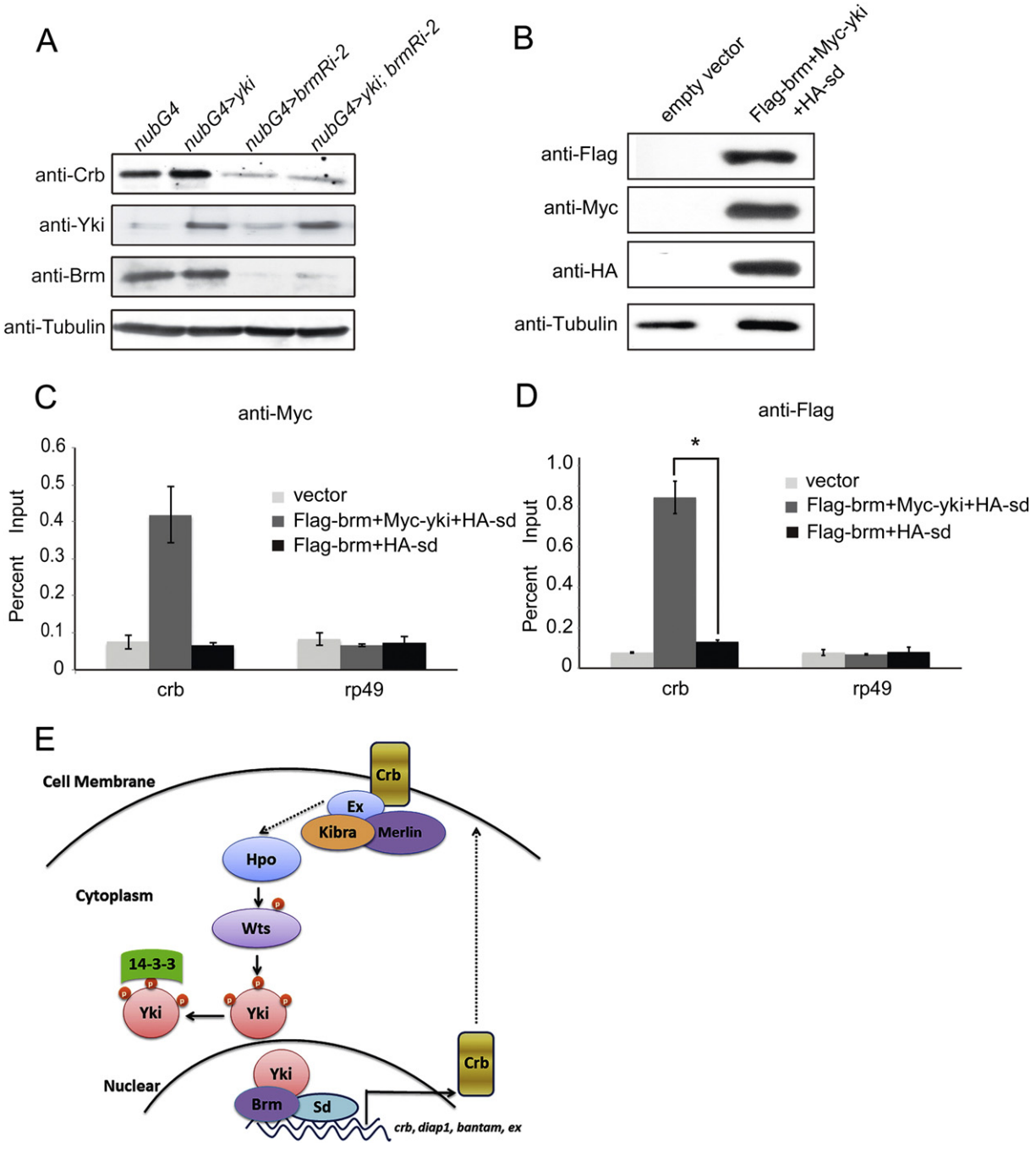

Fig. 5. Yki and Brm bind to the promoter of *crb in vivo*. (A) Third instar wing discs of indicated transgenic flies were extracted to perform western blots. Blots were probed with anti-Crb, anti-Yki, anti-Brm or anti-tubulin antibodies. Note that the overexpression of *yki* elevated the protein levels of Crb, which was greatly repressed by *brm* knockdown. (B) S2 cells were transfected with the *Arm-Gal4, UAS-Flag-brm*, *UAS-Myc-yki* and *UAS-HA-sd* plasmids or control vectors respectively. Lysates were used for Western blotting with anti-Flag, anti-Myc, anti-HA or anti-Tubulin antibodies. (C–D) ChIP assays were performed using anti-Flag or anti-Myc antibodies. Immunoprecipitated DNA was amplified by real-time PCR using primers corresponding either to the promoter region of *crb* or to *rp49* as a negative control. The amount of amplified DNA relative to the input DNA is shown (each bar represents the mean ± SD, $n = 3$, $^*P < 0.01$). (E) Model showing Brm as a novel cofactor for Yki. Brm is important for Crb-regulated Yki activation. Brm–Yki–Sd complex regulates the transcription of Crb in a feedback manner.

altered by Brm knockdown, suggesting Brm does not affect the expression of Yki or Sd.

3.4. Crb forms a feedback loop with Yki dependent on Brm

Previous studies demonstrated that Crb acts as a critical upstream input of Hippo signaling [23,25,26]. We asked whether Brm is required for Crb-driven growth. We found that the overexpression of crb^{intra} causes significant overgrown wings (Fig. 4B), and interestingly, the depletion of *brm* suppressed the overgrowth phenotype (Fig. 4B′ and C). Consistently, the overexpression of crb^{intra} resulted in the upregulation of *ex-lacZ* and enlarged compartment size (Fig. 4D), while knockdown of Brm suppressed the elevated Ex-LacZ levels and the overgrown phenotype (Fig. 4E–E′). To determine whether Crb regulates the expression of *brm*, we generated *crb* non-allele mosaic clones. There is no difference on Brm protein levels was observed between inside and outside clones (Supplementary Fig. 3A–A″), suggesting that Crb does not regulate *brm* expressing. Together, these results indicate that Brm is required for Crb-driven Yki activation.

Interestingly, we found that the overexpression of *yki* increased the levels of Crb (Fig. 4F–F″) and *brm* knockdown obviously mitigated the elevation of Crb induced by *yki* overexpression (Fig. 4G–G″). Consistently, the Crb levels can be decreased by *brm* knockdown (Fig. 4H–H″). Further, the third instar wing discs of indicated transgenic flies were extracted to perform western blots. We observed similar phenotype with the immunostaining (Fig. 5A). These data suggest that *crb* is one of the targets of Yki, and its expression depends on Brm. Further, chromatin immunoprecipitation (ChIP) assay showed that Brm or Yki occupied on the promoter region of *crb* transcription (Fig. 5B–D). The overexpression of *yki* increased the occupancy of Brm on *crb* promoter (Fig. 5D). Together, these data unraveled a feedback loop between Crb and Yki, which is dependent on Brm.

4. Discussion

The core signaling cascade of Hippo pathway has been extensively studied. However, the regulatory mechanism of Yki/YAP activation remains largely elusive. In this study, we found that Brm, a component of SWI/SNF complex, interacts with Yki and regulates organ growth in *Drosophila*. Our findings indicated that Brm is indispensable for Yki activation to drive the expression of target genes. Interestingly, we also found that the expression of *crb* is regulated by Yki–Brm complex. The ChIP assay showed that Brm and Yki physically bound to the promoter region of *crb*, and knockdown of Yki reduced the binding of Brm to *crb* promoter. Taken together, we present a novel feedback loop between Crb and the Yki–Brm complex. Thus, the mutual regulation between apical polarity and Hippo pathway could be critical for tissue growth and homeostasis.

Considering the transcriptional co-dependence with RNA Polymerase II and broad localization in actively transcribed regions [33–35], the Brm complex may be present on multiple promoters and is required by global gene transcription. However, the expression of only 872 genes has been observed significantly altered by Brm knockdown [36]. This suggests that there is a selectivity of Brm-mediated transcriptional regulation. In addition to Hippo pathway, morphogens, such as Wingless (Wg), Hedgehog (Hh) and Decapentaplegic (Dpp), also play fundamental roles in organ patterning and growth. Therefore, we examined the readouts of Wg, Hh and Dpp pathway in wing discs with *brm* knockdown. Interestingly, only Wg secretion was found to be altered (Supplementary Fig. 2A–D″), which argued that there was gene specificity of Brm-mediated transcription. Many other factors including the interaction of transcription factors and co-factors, histone modifications and DNA methylation have been also shown essential in this process [37,38]. Therefore, how Brm regulates the specific targets transcription needs to be further investigated.

In this study, our data demonstrate that the enhancement of Crb levels caused by *yki* overexpression can be suppressed by *brm* knockdown. However, the overexpression of Brm alone cannot alter the Crb levels (Supplementary Fig. 1B–B″′). These results suggest that the regulatory function of Brm complex is indispensable for *crb* but relies on Yki. Together with the fact that Brm physically associate with Yki, we argue that Brm complex is recruited to target gene promoters through Brm-Yki interaction. Accordingly, another *Drosophila* transcription factor Zeste has been reported that recruits the Brm complex to chromatin to initiate transcription [39,40]. Beside of Brm, Moria (Mor), another subunit of the complex, has also been reported to directly interact with Yki and occupy on the promoters of Hpo target genes [10], which support our working model that Yki facilitates the recruitment of Brm complex onto *crb* promoters. Taken together, we draw a conclusion that the specificity of Brm for regulating Hippo targets is determined by Yki recruitment. Given that both the Brm complex and Hippo pathway are conserved in mammals, our results shed a light on a conserved interaction, which might be critical for mammalian growth and tissue homeostasis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2014.12.002.

Author contributions

P.Z. and Z.Y. conceived the project and designed the experiments. The experiments were performed by P.Z., Y.Z., D.L., Y.W. and C.P. The data were analysed by P.Z., Y.Z. and D.L. S.L. and L.Z. provided reagents and comments. Z.Y. Provided critical reading of the manuscript. P.Z. wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests.

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